Development of HPLC Methods for the Purification and Analysis of Plasma Membrane Glycoproteins

by Doris Renauer,* H. Uwe Gierlich,† and Klaus K. Unger†

High resolution HPLC techniques such as affinity chromatography (AC), ion exchange chromatography (IEC), and size exclusion chromatography (SEC) were used successfully for separations of hydrophobic plasma membrane glycoproteins. We have tested a lot of commercially available columns for IEC and SEC and performed the purification of the crude plasma membrane extract with the most suitable columns. By using immobilized ligands with different specificities and sequential affinity chromatography, it is possible to obtain a preliminary structural characterization of the interesting carbohydrate residues of membrane glycoproteins.

Introduction

Glycoproteins and glycolipids constitute the class of glycoconjugates. Most membrane proteins are glycosylated; glycoproteins are widely distributed in animals, plants, microorganisms, and viruses. It has been established that glycans play important biological roles, e.g., the protection of the peptide chain against proteolytic attack; the stabilization of protein conformation; the increase of the immunogenicity of proteins; and the recognition and association with viruses, enzymes, and lectins (1). In addition, plasma membrane-located glycoproteins play a role in processes such as cell adhesion, development, differentiation, tumorigenesis and metastasis, and intercellular recognition and adhesion (2–6).

Glycoprotein Structure

It has been demonstrated that, although glycoproteins contain only a few different monosaccharides, they vary markedly in their carbohydrate content, in the number and degree of branching of their carbohydrate units, and in the distribution of such units along the polypeptide chains. The glycoproteins may be divided into families with similar structures and common oligosaccharide sequences.

As opposed to the O-glycosyl glycoproteins, the N-glycosyl glycoproteins, the class we are interested in, have a common biosynthetic pathway, which leads to at least three different types of structures: the high-mannose type, the hybrid type, and the complex-type glycans, including bi-, tri-, tetra-, and pentaantennary glycans (1,7).

Comparison of Normal and Transformed Cells

Normal diploid cells grow *in vitro* until they form a closed monolayer. The growth is thought to be regulated by specific cell-cell interactions. We have evidence that specific plasma membrane glycoproteins are involved in the contact-dependent inhibition of growth and that the glycan moieties play a crucial role (8). Transformed cells, in contrast, have lost the contact-dependent inhibition of growth and proliferate independently of the actual cell density. Besides this difference, particular biological properties of cells derived from malignant tumors and transformed cells are different from those of nonmalignant or nontransformed counterparts, e.g., they express altered glycan structures of cell membrane glycoproteins (9,10).

Many efforts have been made to elucidate the structural changes of plasma membrane glycoproteins (11–13). Some important aspects are summarized: tumor cells express glycoproteins with increased sizes, the oligosaccharides are more highly branched on the trimannosyl core, the fucose content seems to be higher, and an increased sialylation is found. It is still not clear whether these alterations are causally related to tumorigenesis or to the different behavior of tumor cells.

*Institute of Toxicology, Johannes Gutenberg University, D-6500 Mainz, Federal Republic of Germany.

†Institute of Inorganic Chemistry and Analytical Chemistry, Johannes Gutenberg University, D-6500 Mainz, Federal Republic of Germany.

Address reprint requests to K. K. Unger, Institute of Inorganic Chemistry and Analytical Chemistry, Johannes Gutenberg University, D-6500 Mainz, Federal Republic of Germany.

Experimental Procedures

To clarify the molecular mechanisms of the important cellular phenomena in which glycoproteins are involved and to study the changes in cell-surface characteristics, it is necessary to elucidate the structures of these membrane glycoconjugates. However, in most cases, membrane glycoconjugates are very difficult to isolate in sufficient quantities for structural studies by conventional methods.

For our studies we have used several types of cells: human diploid embryonal lung fibroblasts (FH 109); human osteosarcoma cells (HT 1080); and SV-40 transformed human embryonal lung fibroblasts (WI 38). The plasma membranes were isolated by incubation of the cell monolayers with N-ethyl-maleimide (14). The plasma membrane proteins were extracted with the zwitterionic detergent 3[(3'-cholamidopropyl)dimethylammonio]-1-propane-sulfonate (CHAPS).

Affinity Chromatography

The affinity chromatography studies described here are based on a cooperation with the group R. J. Wieser (15). Immobilized lectins can be used for the isolation of glycoconjugates by affinity chromatography (AC) and for the determination of structural composition of glycoconjugates (16). We have used four lectins and two low-molecular weight ligands with different specificities for glycoproteins.

Concanavalin A (Con A) has weak affinity for the biantennary complex-type and strong affinity for high-mannose and hybrid-type structures. Hybrid-type structures, especially with the bisecting GlcNAc (N-acetylglucosamine) and sialic acid have affinity to wheat germ agglutinin (WGA). Ricinus communis I (RCAI) binds preferentially to terminal β-galactosyl residues. Ulex europeus agglutinin I (UEAI) has affinity for fucosylated glycoproteins (1,16). m-Aminophenylboronic acid (PBA) recognizes 1,2 cis-diols, therefore glycoproteins have affinity toward PBA (17). For binding to serotonin, 5-hydroxytryptamine (Sero), the presence of sialic acid is essential for the interaction (18).

Various methods for coupling proteins to insoluble adsorbents, e.g., silica, can be used for the preparation of immobilized lectins (16). Among the manifold methods of activating silica supports, 3-isothiocyanatopropyltriethoxysilane (ITCPS) was used as the reagent. Covalent binding of lectins or ligands, respectively, was accomplished by reacting the NCS-group of the activated carrier with the amino groups of the proteins or the ligands at physiological pH at 4°C (19). The advantage of coupling the lectins in the presence of inhibiting carbohydrate residues is that the amount of active lectin that is accessible for glycoproteins is much higher. The silica-bound lectins were found to display the same oligosaccharide specificity as lectin-agarose conjugates. They are stable, yielding reproducible elution patterns with extensive usage and time (> 1 year).

For each column, the separation conditions including binding, elution, and in some cases (PBA and Sero), regeneration, were established. The high density of coupled lectins and the separation of native glycoproteins rather than glycopeptides or oligosaccharides required more stringent conditions than those typically required in, for example, lectin-agarose AC. In addition to the specific binding of glycoproteins to lectins, nonspecific protein-protein interaction occurred due to the hydrophobic nature of the proteins. Therefore, relatively high amounts of detergent in binding and elution buffers and an additional step for eluting the nonspecifically bound glycoproteins were necessary. The glycoproteins specifically recognized by the lectin or PBA were eluted from the column by adding the inhibiting sugar, in the case of serotonin by increasing the salt concentration (20, Renauer et al., in preparation). The specificity and the quality of the separations were demonstrated by: a) chromatographic criteria, like rechromatography of the individual fractions, separation in the presence of a sugar not specific for the lectin, and separation in the presence of the inhibiting sugar; b) chromatography of glycoproteins with defined structures; c) sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) (21) (the different fractions show protein patterns differing in concentration and molecular weight); and d) enzyme-linked lectin assay (ELLA) (22) with which the enrichment of carbohydrate sequences can be determined qualitatively and semiquantitatively.

By separating plasma membrane proteins isolated from normal cells grown to confluency (nongrowing cells) from semiconfluent cell cultures (growing cells) and transformed cells on the various affinity columns, a characterization of the glycoprotein composition can be established (Renauer et al., in preparation).

Sequential Affinity Chromatography

To get further information about the differences in the carbohydrate residues of the membrane glycoproteins of different cell types, we have applied sequential or serial affinity chromatography. As originally proposed by Cummings and Kornfeld (23), a mixture of asparagine-linked sugar chains can be systematically fractionated by serial lectin-agarose affinity chromatography. We have adapted the conventional affinity mode to the high performance mode and have fractionated glycoproteins according to their carbohydrate structures by use of a separation scheme that includes four columns with different specificities (Fig. 1).

On the Con A column, the N-glycosidically bound glycoproteins were separated into three fractions: the complex-type structures, the biantennary structures, and a fraction that includes the high-mannose and hybrid-type structures. The hybrid-type glycoproteins were separated on the WGA column from those glycoproteins with high-mannose structures. The fractions with complex-type and biantennary glycoproteins were further fractionated on the RCA I-column to separate the glycoproteins with terminal galactose residues. The unretarded fraction was applied to the Sero column to
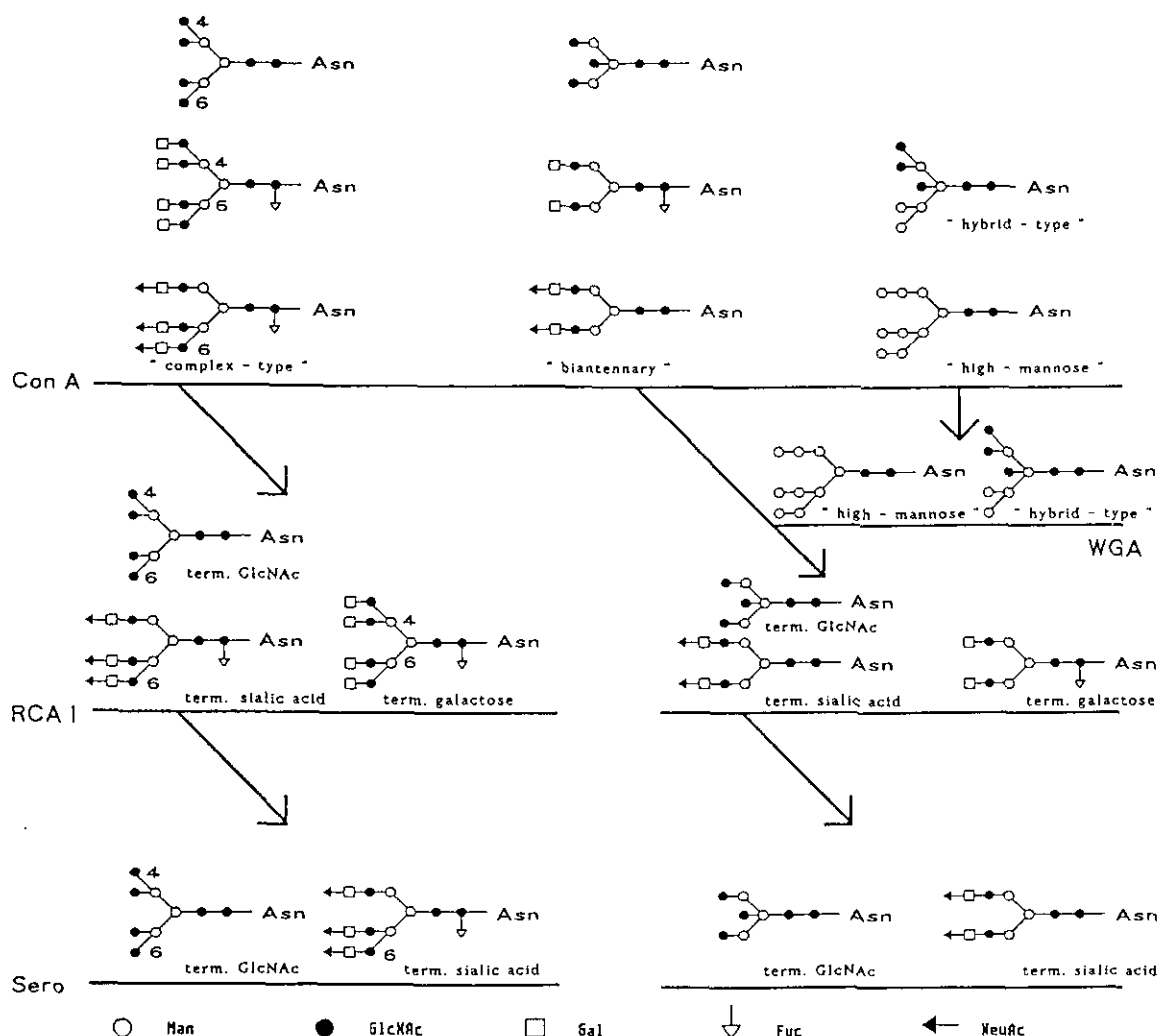
FIGURE 1. Sequential affinity chromatography.

isolate the glycoproteins with terminal sialic acid residues. Between the chromatographic runs on the different columns, a desalting step is necessary to remove detergents and the inhibiting sugars. For determination of the structural characteristics of the different cells, these sequential chromatographic runs were performed with glycoproteins from nongrowing, normal cells, growing normal cells, and transformed cells (Renauer et al., in preparation).

Size Exclusion Chromatography

The extract of type FH 109 was further subjected to size exclusion chromatography to achieve a mild separation according to the size of analytes. Therefore, six commercially available SEC-columns with different particle and pore sizes were tested: from TOSOH (Tonda, Japan): TSK G 2000 SWXL, TSK G 3000 SW, TSK G 3000 SWXL, and TSK G 4000 SW; and from DuPont de Nemours (Wilmington, DE): Zorbax Bio Series GF 250 and Zorbax Bio Series GF 450. To examine the chromatographic prop-

erties of the columns, two sets of experiments were carried out (24): the elution volume, V_e, of standard proteins was measured as a function of the salt concentration (0, 100, 200, 300, 400, or 500 mM NaCl) of the buffered eluent (phosphate buffer, 50 mM, pH 7.0) at constant pH, and the plate height, H, of standard proteins was measured as a function of the flow rate of the eluent (phosphate buffer, 50 mM, pH 7.0 + 300 mM NaCl) at constant mobile phase composition.

The separation of the extract of membrane glycoproteins (FH 109) was then carried out under conditions giving optimal resolution. The best results were obtained by using the Zorbax Bio Series GF 250 column, yielding a separation into two main and five smaller fractions.

Ion Exchange Chromatography

Ion exchange chromatography (25–28) of the type FH 109 extract was carried out on the strong anion exchange column Pro Pac PA 1 (Dionex, Idstein, FRG) specially tailored for high resolution and high capacity

separations of proteins, glycoproteins, and nucleic acid residues. Weak basic chromatographic conditions (pH 8.0) yield a complex chromatogram.

After digesting the protein part of the extract with Pronase E, the carbohydrate residue was examined by using a Carbo Pac PA 1 column (Dionex, Idstein, FRG). Under strong basic chromatographic conditions (pH 13.0) and the application of pulsed amperometric detection (29), neutral oligosaccharides of low chain length could be detected.

Conclusions

The great advantages of high performance AC compared to conventional AC are the short running times and the high recovery rates. The chromatographic runs are completed in less than 90 min with recovery rates of 85 to 95%. It was demonstrated that it is possible to fractionate membrane glycoproteins on immobilized lectins under nondenaturating conditions with reproducible binding and elution patterns during extensive usage and time. The use of ITCPS as an activating reagent allows the coupling of proteins at physiological pH under mild conditions without loss of their biological activity. Although the columns are very small (50 × 7 mm), this method is useful for the separation of semipreparative amounts of glycoproteins due to the high binding capacities of the lectins. The use of a series of different columns has enabled us to fractionate oligosaccharides, glycopeptides, or glycoproteins into structurally distinct groups and to obtain an indication of their structure, which make the subsequent structural studies, such as anion exchange and size exclusion chromatography, much easier. One of our further aims is to clarify the question of whether the structural differences in the carbohydrate composition of glycoproteins are causally related to functional alterations of tumor cells.

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